Arch Biol Sci. 2021;73(3):415-423 https://doi.org/10.2298/ABS210815035H

Chidamide modulates proliferation, migration and apoptosis of human tongue squamous carcinoma SCC9 cells through multiple signaling pathways

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Received: August 15, 2021; **Revised**: August 28, 2021; **Accepted**: August 29, 2021; **Published online**: September 10, 2021

Abstract: Chidamide, a histone deacetylase (HDAC) inhibitor, displays antitumor activities in different tumor cells. Tongue squamous cell carcinoma (TSCC) is the most prevalent oral cavity malignancy with a high incidence and a high mortality rate. We describe the antitumor effects of chidamide on human TSCC SCC9 cells, which has not been reported before. Cell viability and wound healing assay and flow cytometry analysis were used to determine the proliferation, migration, cell cycle and apoptosis of chidamide-treated SCC9 cells *in vitro*. Western blotting was used to detect relative changes in protein levels. Our results reveal that chidamide inhibits SCC9 cell proliferation by decreasing ERK1/2 and mTOR phosphorylation and arresting the cell cycle in G0/G1 phase. Chidamide decreased cell migration in dose- and time-dependent manner by increasing E-cadherin expression. Chidamide induced SCC9 cells apoptosis by increasing the level of cleaved caspase-3 and decreasing the expression of Bcl-2**.** To sum up, chidamide displayed potent antitumor effects on SCC9 cells through multiple signaling pathways and has the potential to be developed as a new therapeutic agent to treat TSCC.

Keywords: chidamide; tongue squamous cell carcinoma; apoptosis; epigenetic modulation; cell migration

INTRODUCTION

Oral cancer is one of the most common malignancies in head and neck cancer, of which 90% is squamous cell carcinoma [1]. Tongue squamous cell carcinoma (TSCC) is the most prevalent oral cavity malignancy, accounting for one-third of oral cancers worldwide [2,3]. TSCC is prone to local invasion and occult lymph node metastasis due to its highly aggressive character which leads to a poor prognosis. At present, surgery combined with radiotherapy and chemotherapy are the main strategies for treating TSCC. Considering the low five-year survival rate [4], it is necessary to develop new drugs for TSCC treatment.

Chidamide (CS055/tucidinostat/HBI-8000), a benzamide-based HDAC inhibitor, was discovered and developed by Chipscreen Biosciences (Shenzhen, China). Chidamide selectively inhibits histone deacetylation activities of HDAC1, 2, 3, 10 [5-7]. HDACs are a group of enzymes that remove the lysine residues in the N-terminal tails of core histone proteins in the nucleosome, regulating chromatin structure remodeling and transcription factor access. During these processes, HDACs affect cell growth and differentiation [8]. Studies indicate that chidamide can suppress cell growth and induce apoptosis in different carcinoma types, such as myelodysplastic syndromes (MDS), human colon cancer and hepatocellular carcinoma, through different cellular signal pathways [8-13]. It was reported that chidamide induces cell apoptosis by upregulating the expression of cleaved caspase-3 and the ratio of Bax/Bcl-2 in MDS and acute myeloid leukemia (AML) cells [9]. Moreover, chidamide affects the mitogen-activated protein kinase (MAPK)/RAS oncoprotein and the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) AKT kinase pathways

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in human colon cancer cells, which results in cell cycle arrest in the G1 phase and apoptosis enhancement [8]. Additionally, as a novel oral class I HDAC inhibitor, chidamide was approved by the Chinese Food and Drug Administration (CFDA) in 2015 for treating patients with recurrent or refractory peripheral T-cell lymphoma (PTCL) and pancreatic cancer [14,15]. It acts as a genuine epigenetic modulator that induces cell apoptosis and inhibits cell growth [16]. These findings prompted us to investigate the possibility of applying chidamide in TSCC therapy.

Herein, we presented the antitumor effect of chidamide in TSCC cell line SCC9. We established that chidamide could inhibit the proliferation and migration of TSCC cells, and induce cell apoptosis through different signaling pathways, indicating that chidamide possesses the potential to be developed as a therapeutic agent with a multiple targets strategy for treating TSCC.

MATERIALS AND METHODS

Cell culture, chemicals and reagents

Human TSCC SCC9 cells were grown in Dulbecco's modified eagle's medium (DMEM) (HyClone Laboratories Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, USA), 100 U/mL penicillin and 100 μg/mL streptomycin sulfate at 37°C in a 5% CO_2 incubator. Chidamide was purchased from Chipscreen Biosciences, (Shenzhen, China), and was dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) to a 2 mM stock solution. When SCC9 cells grew to 90% confluence, they were treated with different concentrations of chidamide for 48 h. Antibodies against cleaved-caspase-3 (#9662), Bcl-2 (#2872), Bax (#2772), β-catenin (#8480), β-actin (#3700) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against signal transducer and activator of transcription 3 (STAT3) (A11216), phosphorylated mammalian target of rapamycin (p-mTOR) (AP0094) and phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) (AP0472) were purchased from ABclonal Technology (Woburn, MA, USA). Antibodies for mTOR (20657-1-AP) and ERK1/2 (16443-1-AP) were purchased from Proteintech Group, Inc. (Wuhan, China), E-cadherin (abs130068) was purchased from Absin Bioscience Inc. (Shanghai, China). The antibody for phosphorylated STAT3 (p-STAT3) (381552) was purchased from Zen Bio Science (Chengdu, China).

Cell proliferation analysis by the CCK8 assay

SCC9 cells were seeded in a 96-well plate (1×10^4 cells per well) and incubated overnight. The cells were treated with different concentrations of chidamide (0, 1.25, 2.5, 5, 10 and 20 μM) for 24 h, 48 h and 72 h. After treatment, the medium in each well was replaced with 100 μL CCK8 solution (containing 10 μL CCK8 regent and 90 μL DMEM basic medium), and incubated at 37°C for 30 min. The absorbance at 450 nm (A_{450}) of each well was measured, and the growth inhibition rate was calculated as described as follows:

$$\text{Growth inhibition (\%)} = 1 - (A_{450} \text{ of treat group} - A_{450} \text{ of blank})/(A_{450} \text{ of control group} - A_{450} \text{ of blank})\times 100\%.$$

The IC_{50} value of chidamide in SCC9 cells was calculated by SPSS software.

Cell cycle analysis by flow cytometry

Chidamide-treated cells were harvested and washed with phosphate buffered saline (PBS), and the cells were suspended and fixed in 70% ethanol overnight at 4°C The fixed cells were treated using a Cell Cycle analysis kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's protocols. After staining with propidium iodide (PI), flow cytometry analysis was conducted using a CytoFLEX flow cytometer (Beckman, USA) to detect DNA content. Quantification of cell cycle distribution was analyzed using FlowJo Version 10 software.

Wound healing assay

Cells were seeded in a 6-well plate and cultured to 70-80% confluence; a straight line was scratched by a pipette across the cell layer in each well and washed gently with PBS twice. The cells were treated with 0, 1.25, 2.5 and 5 μM chidamide, and the width of the

'wounds' was measured at different time points under a microscope (Olympus IX71, Japan).

Apoptosis analysis by flow cytometry

SCC9 cells were treated with 0, 1.25, 2.5 and 5 μM chidamide for 48 h. The cells were washed with PBS twice and resuspended in binding buffer and stained using the Annexin V FITC/PI kit (Best Bio Science, Shanghai, China) according to the manufacturer's protocol. Apoptosis was evaluated using the flow cytometer. The percentage of apoptotic cells in the total number of cells was counted by software.

Western blotting analysis

Cells were incubated in a 6-well plate (1×10^6 cells per well) at 37 °C, 5% CO_2 overnight and then treated with different concentrations of chidamide (0, 1.25, 2.5, and 5 μM) for 48 h. Cells were harvested and lysed in 100 μL RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Beyotime Biotechnology, Shanghai, China), protease inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitor (Roche). The protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins were separated by12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA), blocked with 5% non-fat milk. Membranes were subsequently incubated with the primary antibodies overnight, then washed and incubated with goat anti-rabbit secondary antibody (AS014, ABclonal Biotech,). Protein bands were visualized using a SuperSignal™ West Pico PLUS Chemiluminescent substrate (Thermo Fisher Scientific) on a gel-imaging system (GE Healthcare, Chicago, IL, USA).

Statistical analysis

Statistical differences were determined using one-way ANOVA followed by Bonferroni's post hoc test for multiple groups. Statistical significance was $P<0.05$ or as indicated. All data are displayed as the mean±SD (standard deviation), which was analyzed using GraphPad Prism 6.0 (GraphPad Software, Inc.). All experiments were performed at least three times independently.

RESULTS

Chidamide inhibited SCC9 cells proliferation and arrested the cell cycle

To determine whether chidamide suppresses the proliferation of SCC9 cells, the cells were treated with 0, 1.25, 2.5, 5, 10 and 20 μM chidamide for 48 h, and changes in cell morphology were observed under a microscope. The shape of chidamide-treated cells changed in a dose-dependent manner compared with the control (treated with solvent without chidamide). Adhesion ability declined at low concentrations of chidamide (1.25 and 2.5 μM); with increasing concentrations (5, 10 and 20 μM), most of the cells began to float and die (Fig. 1A). The viability of treated cells was determined by the CCK8 assay. SCC9 cells were treated with different concentrations of chidamide for 24, 48 or 72 h. The results showed that chidamide suppressed cell proliferation in a dose- and time-dependent manner (Fig. 1B). The IC_{50} of chidamide in SCC9 cells was 5.497 μM at 72 h, and 7.849 μM at 48 h. Both values are consistent with the IC_{50} value range reported in other carcinoma cell lines [11].

To further investigate the effect of chidamide on cell proliferation, cell cycle analysis showed that the cells accumulated in the G0/G1 phase in a dose-dependent manner after treatment with chidamide for 24 h (Fig. 1C and D), which suggested that cell cycle arrest might be an important cause of cell proliferation inhibition in chidamide treatment.

Chidamide suppressed TSCC cell proliferation by decreasing ERK and mTOR phosphorylation

To elucidate the underlying molecular mechanisms of the inhibitory effect of chidamide on TSCC cell proliferation, we investigated whether the Janus kinase (JAK)/STAT and MAPK/ERK signaling pathways were involved in this process, as both were reported to participate in chidamide-induced cell growth inhibition [8,17]. STAT3 and ERK were detected by Western blotting (Fig. 2A). Basal ERK expression was

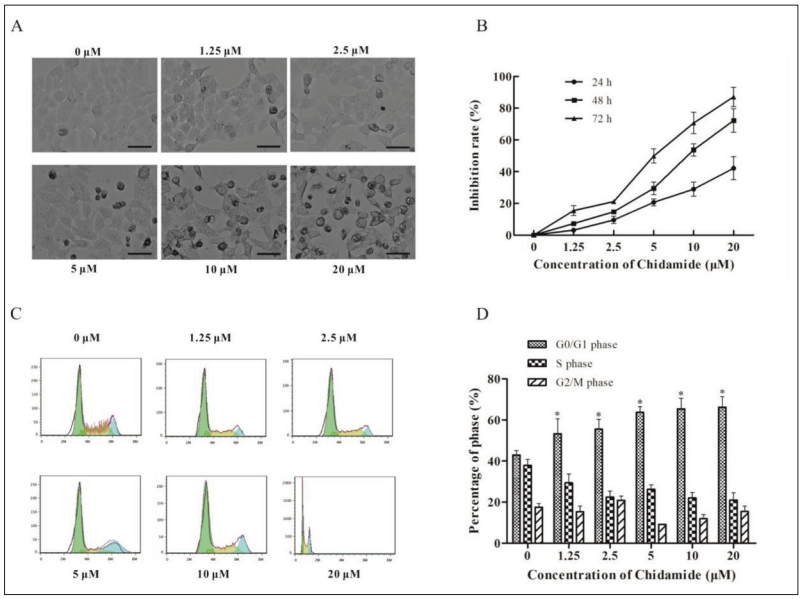

Fig. 1. Chidamide significantly inhibited TSCC cell proliferation. **A –** SCC9 cells were treated with different concentrations of chidamide (0, 1.25, 2.5, 5, 10 and 20 μM) for 48 h. Cell morphology change was examined by microscopy. Scale bar indicates 50 μm. **B –** Cells were incubated in 96-well plates and treated with different concentrations of chidamide for 24, 48 and 72 h. CCK8 solution was added to each well and A_{450} of each well was measured. The inhibition rate was calculated as described. **C –** Cells were cultured to 70-80% confluence and treated with different concentrations of chidamide for 48 h. The cell cycle was analyzed by flow cytometry. **D –** Data analyzed by FlowJo software; the cells at different phases were counted and the result is represented in the bar graph. * indicates P<0.05 compared with the 0 μM of the G0/G1 phase group.

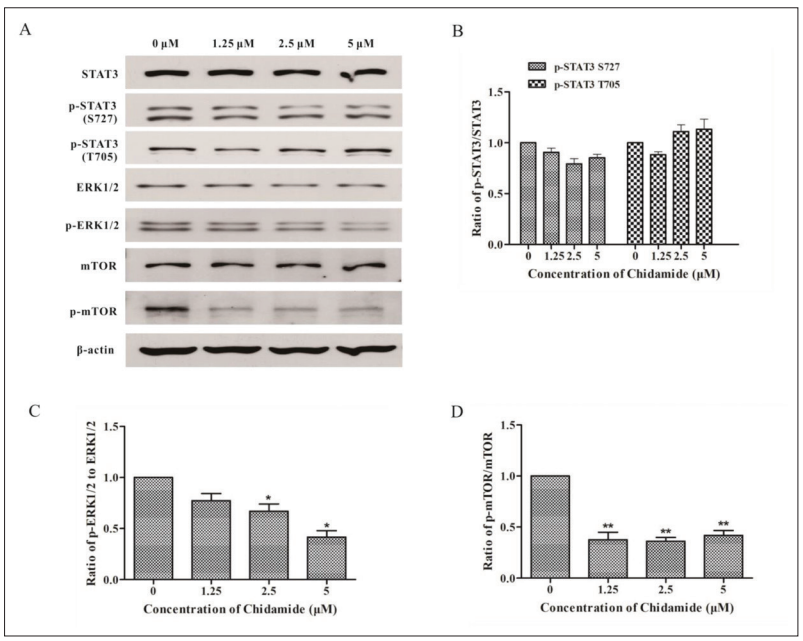

Fig. 2. Chidamide suppressed the proliferation of SCC9 cells through ERK and mTOR signaling pathways. **A –** Western blotting results of proliferation related protein expression in SCC9 cells treated with different concentrations of chidamide. **B to D –** The OD value of Western blotting bands was analyzed using Image J. * indicates P<0.05, ** indicates P<0.01 compared with the 0 μM group.

unchanged in chidamide-treated cells, but the ratio of p-ERK/ERK declined in a dose-dependent manner (Fig. 2C), whereas neither the expression of STAT3 nor the ratio of p-STAT3/STAT3 were affected by chidamide (Fig. 2B).

PI3K/AKT/mTOR is an important pathway involved in many anticarcinogenic processes related to cell growth, survival, metastasis and apoptosis [1]. Western blotting results indicated that p-mTOR dramatically decreased in chidamide-treated cells while the expression of mTOR remained unchanged (Fig. 2D), suggesting that chidamide might influence the phosphorylation process of mTOR.

Chidamide inhibited the migration of SCC9 cells through increased expression of E-cadherin

Cell migration was analyzed by the wound healing assay. Compared to the untreated group, wound closure of chidamide-treated cells was significantly decreased with time (Fig. 3A). At the same time point, wound closure decreased with increasing concentrations of chidamide (Fig. 3B). These results revealed that chidamide could suppress migration of TSCC cells *in vitro*. To elucidate the underlying mechanism, the expression of metastasis-related proteins, E-cadherin and β-catenin, was investigated. Western blotting showed that the expression of β-catenin, which is positively correlated with clonogenic cell growth [18], did not change in chidamide-treated cells (Fig. 3C and D), whereas the expression of E-cadherin, which is negatively related to cell invasion potency [19], was significantly increased in a dose-dependent manner (Fig. 3C and D).

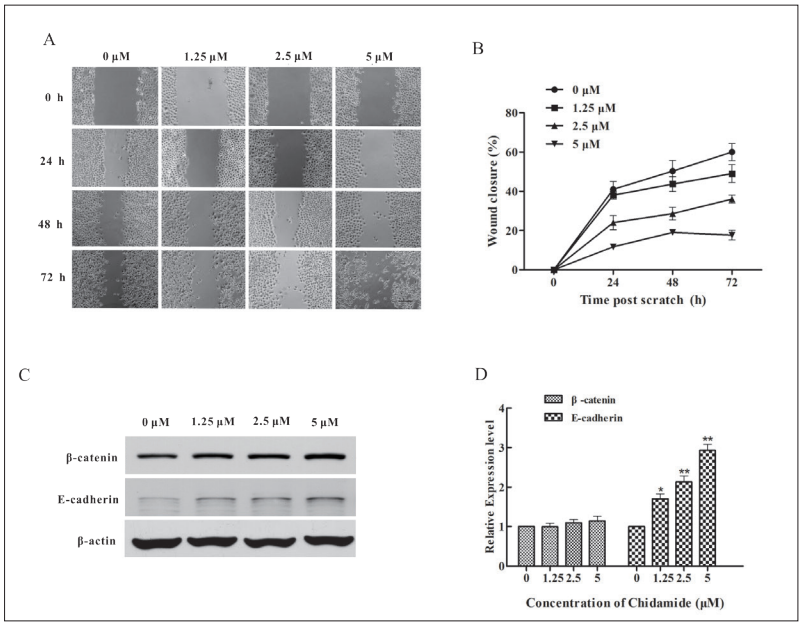

Fig. 3. Chidamide suppressed SCC9 cell migration by increasing the expression of E-cadherin. **A –** Cells were cultured to 70~80% confluence and then treated with different concentrations of chidamide, respectively. Artificial wounds were created and the effect of chidamide on wound closure was evaluated at different time points. Photographs were taken by a digital camera (scale bar indicates 200 μm). All wound healing experiments were performed in triplicate and repeated at least three times. **B –** Wound closure was determined by measuring the width of the wounds on photographic images using digital needlepoint calipers. Data are expressed as the percentage of wound closure compared with the original wound width photographed at time zero; data include results from three separate assays. **C –** Western blot results of migration-related protein expression in cells treated with different concentrations of chidamide. **D –** The bar graphs indicated the expressions of β-catenin and E-cadherin relative to β-actin. * indicates P<0.05, ** indicates P <0.01, compared with 0 μM group.

Chidamide induced SCC9 cells apoptosis through the Bcl-2/caspase-3 pathway

As chidamide was reported to induce apoptosis in various types of cancer cells, we examined apoptosis induction in chidamide-treated SCC9 cells using flow cytometry. As shown in Fig. 4A and B, the percentage of early apoptotic and late apoptotic cells gradually increased with increasing concentrations of applied chidamide. Examination of the expression of apoptosis-related proteins by Western blotting analysis indicated that the expression of Bcl-2 decreased, while the level of cleaved caspase-3 increased after chidamide treatment (Fig. 4C and D), but the expression of Bax did not change significantly. These results suggest that chidamide might induce apoptosis in SCC9 cells through a decrease in the Bcl-2/Bax ratio and increased activation of caspase-3.

DISCUSSION

The prognosis of current treatment of TSCC is not optimistic, and the development of new pharmacological agents is a priority in TSCC treatment. Chidamide, one of the selective inhibitors of HDAC, displayed a remarkable antitumor effect in TSCC cells in this study. HDACs play a vital role in epigenetic abnormalities, which have become an important indicator of tumorigenesis. When HDAC is overexpressed in cells, it causes an imbalance in histone protein acetylation and leads to an open, active chromatin state that affects different cellular processes, including cell survival, homeostasis, cell proliferation and gene expression. As a result, inhibiting HDACs has become an important approach to tumor therapy. The suberoylanilide hydroxamic acid (SAHA, vorinostat) is the first HDAC inhibitor approved by the FDA for cutaneous T cell lymphoma (CTCL) treatment [23]. In recent years, more HDAC inhibitors, including chidamide, have been approved for the treatments of different cancers [24].

Chidamide is a structural analog of MS-275 (entinostat), which is a benzamide derivative with anticancer effects on different types of cancer [25]. Compared to MS-275, chidamide is more stable and can be stored at room temperature. Moreover, chidamide exhibits a longer half-life and is less toxic *in vivo* than MS-275 [8]. There is increasing evidence indicating that chidamide has the potential to be applied in treatments of different types of cancer [5,9-14]. Based on this, we conducted the current study and explored the molecular mechanisms responsible for the antitumor activity of chidamide in TSCC.

Herein, we found that chidamide could modulate SCC9 cell proliferation, migration and apoptosis. To uncover the underlying molecular mechanism, JAK/STAT and MAPK/ERK pathways were examined. In chidamide-treated SCC9 cells, the phosphorylation

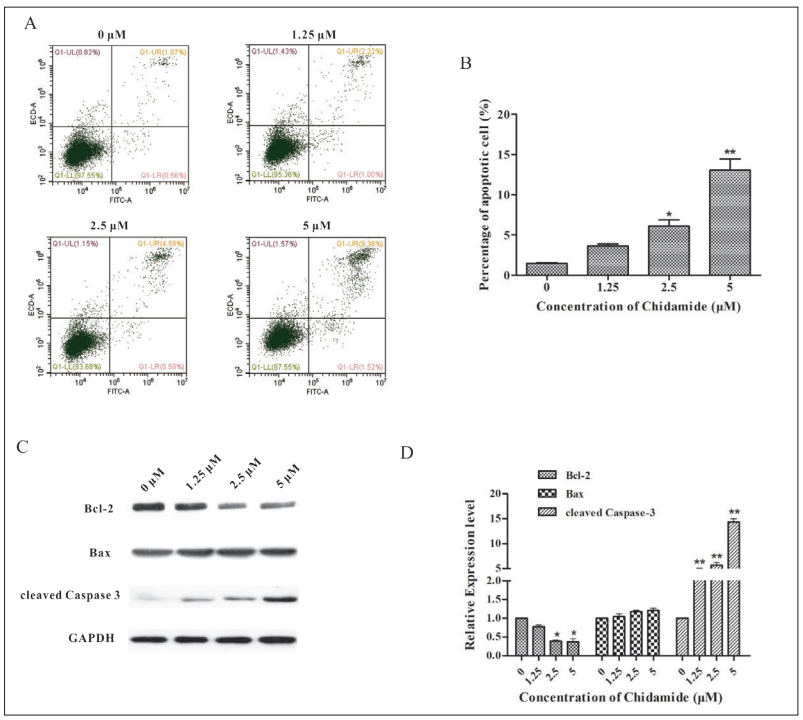

Fig. 4. Chidamide induced SCC9 apoptosis in a dose-dependent manner through the Bcl-2/caspase-3 pathway. **A –** Cells were treated with different concentrations of chidamide for 48 h, and apoptosis of cells was analyzed by flow cytometry. The 0 μM-treated group was used as the negative control. **B –** Apoptotic cells were counted by software and the apoptosis rate is represented in a bar graph. **C –** Western blotting results of apoptosis-related protein expression in SCC9 cells that were treated with different concentrations of chidamide. **D –** The bar graph indicated the expressions of proteins relative to GAPDH. * indicates P <0.05, ** indicates P <0.01.

of ERK1/2 decreased, while the phosphorylation of STAT3 was unchanged, which means the ERK signal might be an important mechanism underlying the tumor-suppressive function of chidamide in TSCC.

The PI3K/AKT/mTOR pathway in cancer contributes to a tumorigenic phenotype through effects on multiple cellular processes such as apoptosis, proliferation, motility, cell transformation [26]. The activation of the PI3K/AKT/mTOR pathway is frequently implicated in resistance to anticancer therapies. It has been reported that the activation of the PI3K/AKT/mTOR pathway could modulate the expression of multidrug resistance-1, a trans-membrane drug transporter implicated in the development of multidrug resistance (MDR) [27]. P-mTOR was reported to be overexpressed in more than half of TSCC patients [28]; moreover, this clinical trial suggested the aberrant expression of p-mTOR might be independently associated with poor prognosis in patients with TSCC [28], and drug resistance might be the reason. Thus, inhibitors of the PI3K/AKT/mTOR pathway are being rapidly evaluated in preclinical models and in clinical studies to determine whether they can restore therapeutic sensitivity when given in combination. Some studies showed that mTOR inhibitor could suppress tumor growth and sensitize a tumor to radiation, cytotoxic agents and epidermoid growth factor receptor (EGFR) inhibitors *in vivo* and *in vitro* [29]. This means that mTOR inhibitors not only exert anticancer activity but also act as a sensitizer to radiotherapy and chemotherapy. Chidamide might act as an effective mTOR inhibitor in SCC9 cells because it displayed a marked inhibitory effect on the phosphorylation of mTOR, even at a low concentration (1.25 μM). Our results propose the potential of chidamide to be applied in personalized adjuvant therapy of TSCC, and more than half of TSCC patients might benefit from the application of chidamide.

Metastasis is a major cause of death in patients with cancer. Invasion of surrounding tissues and metastasis have been proposed to initiate subsequent loss of the intercellular adhesion protein E-cadherin [19]. β-catenin is an adhesion protein that binds with E-cadherin to form a compound regulating cell migration [30]. In SCC9 cells, the expression of β-catenin was not affected by chidamide treatment, while the expression of E-cadherin was upregulated by chidamide in a dose-dependent manner, indicating that chidamide might restrain the initiation of TSCC cell migration by increasing E-cadherin expression.

Induction of cell cycle arrest and apoptosis is also an important antitumor mechanism of HDAC inhibitors. Cell accumulation in the G0/G1 phase of the cycle was observed in chidamide-treated SCC9 cells. The mitochondrial apoptosis pathway plays an important part in cell apoptosis. Bcl-2 suppresses apoptosis by regulating the activities of downstream caspase signals, but Bax has an opposite effect to Bcl-2. Caspase-3

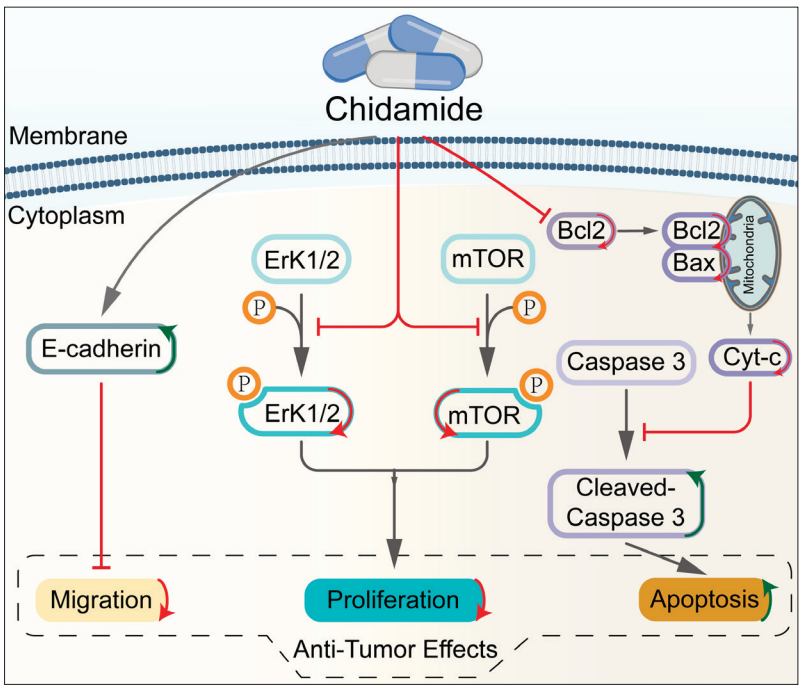

Fig.5. The schematic model of the antitumor mechanisms of chidamide in SCC9 cells. Chidamide upregulates the expression of E-cadherin, inhibits phosphorylation of ERK and mTOR, and decreases the expression of Bcl-2. Through these signaling pathways, chidamide displays an effective antitumor effect on SCC9 cells.

will be activated by increased Bax and downregulation of Bcl-2, which leads to cell death. Chidamide was shown to decrease Bcl-2 and activate caspase-3 in SCC9 cells, which led to cell apoptosis increase even without changing Bax expression. These results are consistent with previous studies of chidamide in other cancer cells [8,9].

In the process of carcinogenesis, crosstalk between different signaling pathways is common, and the inhibition of one signaling pathway can lead to a compensatory activation of another signaling pathway. These compensatory effects might weaken the anticancer effect of drugs with a single target, often leading to multidrug resistance (MDR). Thus, research on therapeutic agents with multiple targets will become very attractive, for multiple oncogenic signaling pathways can be simultaneously targeted increasing the likelihood of overcoming MDR in difficult-to-treat cancer. A recent study demonstrated that chidamide combined with doxorubicin (DOX) could suppress MDR breast cancer cells through synergistically modulation of multiple signaling pathways [31]. In the current study, chidamide was shown to possess an antitumor effect in TSCC *in vitro* through multiple signaling pathways. As summarized in Fig. 5, chidamide inhibits TSCC cells mainly through modulating cell proliferation, migration and apoptosis. First, chidamide reduces SCC9 cell growth and proliferation by phosphorylation of ERK1/2 and mTOR. Through these two signaling pathways, chidamide could not only inhibit cell growth but also enhance TSCC cell sensitivity to other therapeutic agents in combination therapy. Second, it decreases the expression of E-cadherin to inhibit the initiation of SCC9 cell migration, which helps to reduce the possibility of local invasion and lymph node metastasis in TSCC and to improve prognosis. Third, the downregulation of Bcl-2 induced by chidamide reduces the formation of the Bcl-2/Bax heterodimer, which causes mitochondrial release of cytochrome-c to decrease. As a result, more cleaved-caspase-3 is produced, which leads to an increase in cell apoptosis.

In conclusion, this study shows that chidamide could be considered as a new HDAC inhibitor that possesses effective antitumor activity in TSCC cells. Further studies are needed to evaluate the therapeutic role of chidamide *in vitro* and *in vivo* for the treatment of TSCC.

Funding: This work was supported by the National Natural Science Foundation of China (Grant No. 81700446 and No. 82174020); the Foundation of Health Commission of Hubei Province (Grant No. WJ2017M175; WJ2019F133), and the Natural Science Foundation of Hubei Province of China (Grant No. 2012FFB04903).

Author contributions: Hongyao Huang and Tao Deng contributed equally to this work. Hongyao Huang, Tao Deng, JinQian, and Min Tian participated in the experiments and data collection. Jie Hu and Yangyang Zhu performed data analysis and drafted the figures. Xiaohong Guo participated in the conception and design of the study and revised the manuscript. Lili Lu participated in the design of the study, provided administrative support, and drafted the manuscript. All authors approved the final version of the manuscript.

Conflict of interest disclosure: The authors have no conflicts of interest to declare.

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